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Report for DAMD17-97-1-7342

Abstract

During the first year of our grant we have made progress on all three specific aims. First, for specific aim one, we have concentrated on the development of additional Ras effector domain mutants to determine the role of NF1 as an effector of Ras function. Second, for our studies in specific aim two, we have characterized a novel farnesyltransferase inhibitor that may have properties that will render it more effective in our studies to determine if such inhibitors of Ras can effectively block NF1- and NF2-deficient tumor cells. Third, for specific aim three, we have determined that NF2 blocks Ras transformation, in part, by blocking the action of the Rac small GTPase.

Introduction

Although the NF1 and NF2 candidate tumor suppressor genes have been implicated in the development of neurofibromatosis type 1 and 2, respectively, the precise functions of their encoded proteins remain to be elucidated. NF1 encodes neurofibromin, which has been shown to function as a GTPase activating protein (GAP) for the Ras proto-oncogene proteins. However, there is also evidence that neurofibromin may mediate downstream effector signaling pathways that promote Ras regulation of cellular differentiation. Whether neurofibromin is an effector of Ras remains to be determined. NF2 encodes merlin, a member of the ERM family of proteins that are believed to function in linking cytoskeletal components with membrane proteins. However, little is known concerning merlin function. Like neurofibromin, merlin expression has been shown to antagonize Ras transformation. However, how either protein blocks Ras transformation remains poorly understood.

Farnesyltransferase inhibitors (FTIs) have been shown to be potent inhibitors of Ras transformation and are promising drugs for cancer treatment. FTI treatment has also been shown to be effective in blocking the transformed growth of an NF1-deficient malignant schwannoma cell line that exhibits upregulated Ras activity. However, whether FTIs will be effective inhibitors of the majority of NF1-deficient tumors and whether they will be useful for the treatment of NF2-deficient tumors has not been established.

Summary of Progress

Progress this year has been made in several different areas during the past year. In specific aim one, our goal was to assess the possible role of NF1 as an effector of Ras function. In these studies, we have concentrated on the development of additional Ras effector domain mutants to address this question. Effector domain mutants have proven to be one of the most powerful approaches for dissecting the contribution of candidate effectors of Ras function. In specific aim two, one goal was to determine if farnesyltransferase inhibitors (FTIs), developed originally as anti-Ras drugs, can effectively inhibit the uncontrolled growth properties of NF1- and NF2-deficient tumor cells. During the past year, how FTIs actually work has become problematic. Thus, despite a demonstration that they are potent anti-tumor drugs, in xenograft and transgenic mouse tumor models, what they actually target has become a complex issue. Therefore, during the past year, we have assessed the anti-tumor actions of a novel FTI that may

add strength to our analyses. Finally, in specific aim three, we proposed studies to follow up on a published study that showed that NF2 blocked Ras transformation. For this aim, we have now determined that NF2 blocks Ras transformation, in part, by blocking the action of the Rac small GTPase.

Body

In year one we have initiated efforts to work on all three tasks from our statement of work. Progress on Task A (specific aim one), to determine if neurofibromin (NF1) functions as a Ras effector that contributes to its role as a tumor suppressor, will continue into the next year. Progress on Task B (specific aim two) involved the detailed characterization of a novel farnesyltransferase inhibitor (FTI) to compare it to other known FTIs. These studies were aimed at determining which FTIs will be used in the studies proposed in Task B. Progress on Task C (specific aim three), to determine if merlin (NF2) inhibition of Ras is a consequence of antagonizing Ras via blocking Rho family protein function, was significant and will continue into the next year.

A. **Determine if NF1 is an effector of Ras function.**

Key reagents for dissecting the contribution of NF1 as an effector of Ras involve the use of Ras effector domain mutants. Therefore, during the past year, we have concentrated on the development of more effective reagents to assess such a function. First, we have generated additional effector domain mutants, involving single amino acid substitutions, in the Ras effector domain (residues 25-45). Second, we have introduced these mutant sequences into retrovirus expression vectors. This second step has been essential in light of our experience that the transfection efficiency of our previously developed vectors was low, and we were concerned that we were analyzing subclones of transfectable cells. Retrovirus infection has proven to be much more effective for introduction of Ras effector domain mutants into human tumor cells. We have generated several variations of these mutants, using the pLSXN (neo-resistant), pBABE-puro (puromycin-resistant), and pCTV3-HA (hygromycin-resistant) retrovirus vectors. This repertoire of vectors provides us with selectable markers to allow us to perform cotransfection analyses with our NF1 constructs, followed by co-selection in drug-containing media, for cell lines stably expressing Ras and NF1 variants.

B. **Determine if FTIs can block the transformed growth properties of NF1- or NF2-deficient tumor cells.**

Our original proposal described the use of several FTIs for these studies. All of these FTIs work as antagonists of farnesyltransferase by blocking one of its substrates, the CAAX tetrapeptide sequence of Ras. During the past year, a new FTI has been described that blocks farnesyltransferase activity by blocking the ability of its second substrate, farnesylpyrophosphate (FFP), from complexing with the enzyme. These FTIs, provided by Rhone-Polenc Royer, were described to be more effective in their ability to block all three forms of the Ras protein (H-Ras, K-Ras, and N-Ras). In contrast, all of the CAAX-inhibitory based FTIs work effectively only against H-Ras, but not N-Ras or K-Ras. This limited ability posed a possible concern for our studies to target NF1- and NF2-deficient tumor cells, since what Ras protein they may express is

not known. This new FTI represented a potentially more useful inhibitor for our studies. Consequently, we have been comparing the action of this FTI with those of the more conventional CAAX-based inhibitors to determine if this inhibitor will be a superior drug for our studies. Figure 1 shows a representative analyses of these compounds against human tumor cells (we have analyzed a range, including those derived from breast, colon, lung, and pancreas). We wanted to first compare these compounds on tumor cells where we have previously analyzed other FTIs. These analyses were done in cells suspended in soft agar. When compared to the control culture (vehicle), where large soft agar colony formation was seen, all three drugs caused very effective inhibition of growth. These compounds will now be used in our analyses of NF1- and NF2-deficient cells.

C. Determine if NF2 inhibition of Ras transformation is a consequence of antagonizing Ras via blocking Rho-dependent signaling pathways.

In these studies, our first goal was to validate, using our reagents, that NF2 indeed can block Ras transformation. This first step was necessitated by the increasing concerns of cell type differences in Ras function. For these studies, we received a series of different NF2 expression constructs from Dr. Nancy Ratner, that represented various deletion mutants of full length human NF2. As shown in Figure 2, we found that several of these constructs (NF2-I, NF2-II) did effectively block Ras focus-formation when assayed in NIH 3T3 cells. The degree of inhibition seen ranged from 40-50%. This incomplete inhibition is due, in part, to incomplete co-transfection of both Ras and NF2 expression vectors into all cells.

Ras transformation is mediated by activation of multiple signaling pathways that in turn activate a number of nuclear transcription factors. We and others have shown that inhibition of several of these signaling events, such as the activation of the Elk-1 and Jun transcription factors, can block Ras transformation. Therefore, we speculated that NF2 inhibition may be a consequence of inhibition of these pathways. As shown in Figure 3, we indeed found that the NF2 constructs that blocked Ras transformation (Figure 2) were effective inhibitors of oncogenic Ras activation of both Elk-1 and Jun. Elk-1 activation by Ras is mediated through activation of the Raf/MEK/ERK pathway. In contrast, Ras activation of Jun is via a separate pathway, involving phosphatidylinositide 3-phosphate kinase (PI3K) activation of Rac, which in turn activates the Jun N-terminal kinase (JNK), the activator of Jun. The ability of NF2 to block two distinct pathways of Ras signaling was unexpected. Thus, while the inhibition of the Rac/JNK pathway is consistent with our starting hypothesis, that Rho family proteins such as Rac may be the target of NF2, that NF2 can also target the Raf/MEK/ERK kinase cascade was not expected. These result demonstrate that NF2 antagonism will be complex.

Since Ras mutations are not seen in NF2-deficient tumors, the ability of NF2 to antagonize oncogenic Ras is not completely relevant to these tumors. However, endogenous Ras may be upregulated and activated, without mutational activation, in response to extracellular signaling. An autocrine growth loop, where NF2-deficient cells synthesize and secrete growth factors that stimulates their growth, is possible. To address these possibilities, we determined if NF2 can also antagonize signaling to Elk-1 and Jun via serum stimulation or in the absence of stimulation (autocrine growth). As shown in Figure 4, we found that NF2-I and NF2-II both blocked calf serum-induced growth of NIH 3T3 cells. We also found that both blocked upregulation of transcription from the cyclin D1 promoter. This later observation suggests that NF2 function may modulate progression of cells through the G1 phase of the cell cycle. These

inhibitory actions were not due to nonspecific actions, since the NF2 variants did not inhibit activation of serum response factor (SRF).

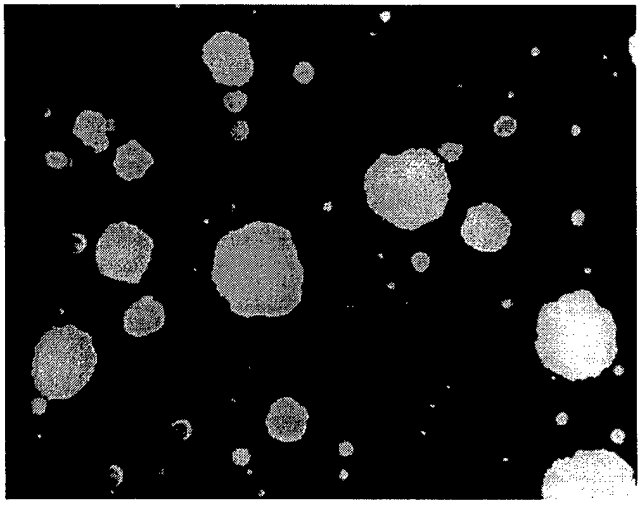
Finally, we also analyzed the ability of NF2 to block growth-stimulatory signals in serum-starved cells. This analysis measures the contribution of basal activities, due possibly to stimulation by an autocrine loop. As shown in Figure 5, we saw a similar pattern of inhibition seen with serum-stimulated cells. However, NF2-I and NF2-II was most effective at blocking cyclin D1 and Jun activation, and no significant inhibition of Elk-1 was seen.

Future Directions

We will continue on all three of our proposed task in the next year. Our studies in specific aims one and two concentrated on reagent development and validation. We are now ready to utilize these reagents for our proposed analyses. Studies in specific aim three will continue to dissect the signaling pathways that are antagonized by NF2 to block growth mediated by oncogenic Ras as well as serum growth factors.

Conclusions

In summary, during the first year we have made advances on all three tasks. As a consequence of new information from our studies and those of others, we have made some revisions to some our proposed approaches. For example, the recent revelations that FTIs may cause a block in tumor growth, not via blocking Ras, has forced us to reevaluate our present inhibitors and to consider the use of more recently developed inhibitors. Our progress on Task C have advanced significantly, and we will pursue these aspects to completion in the second year.



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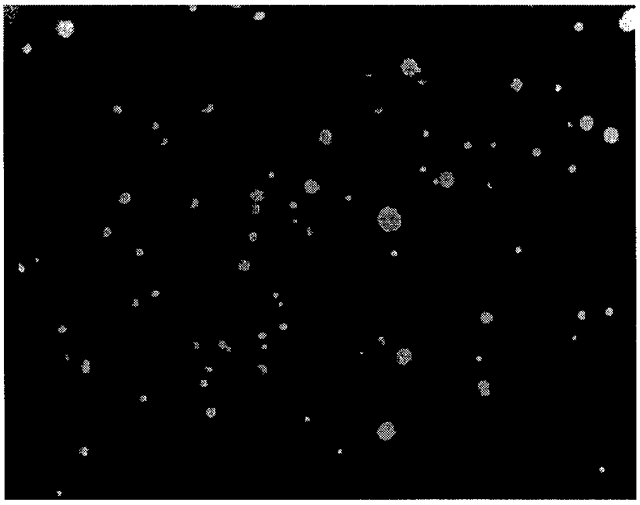

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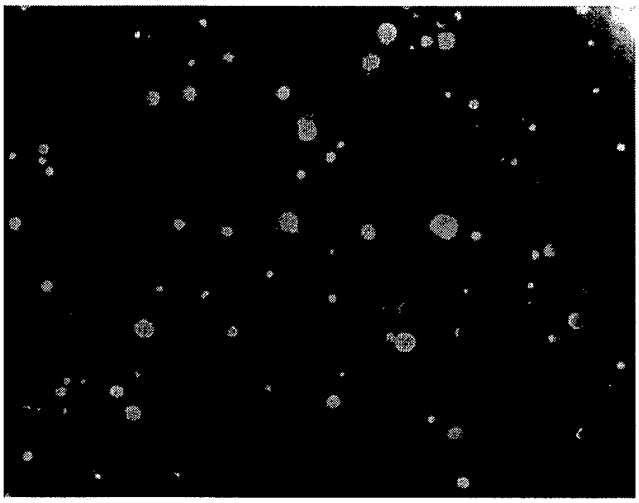

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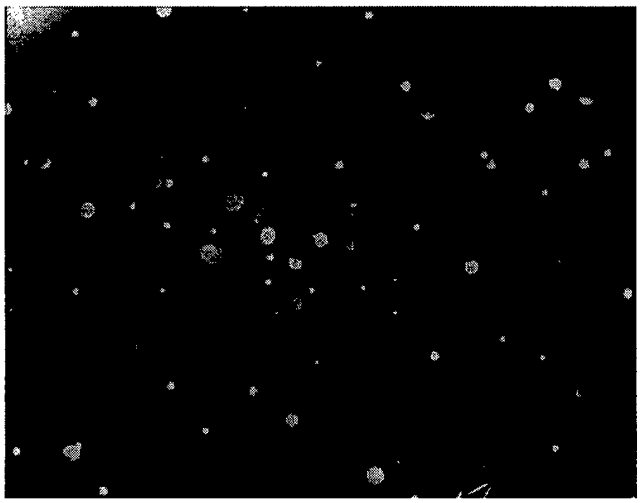

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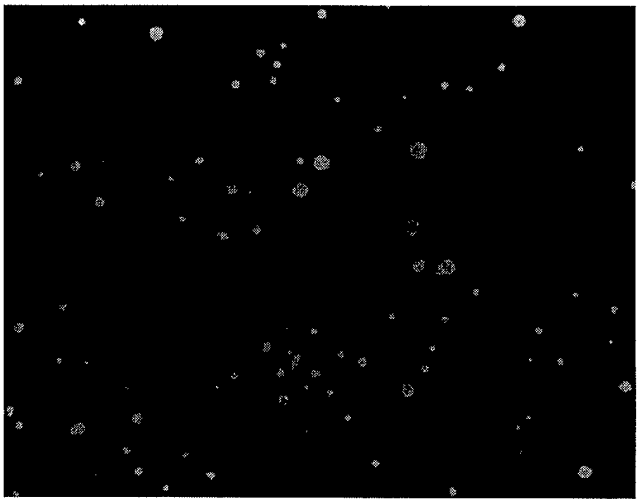

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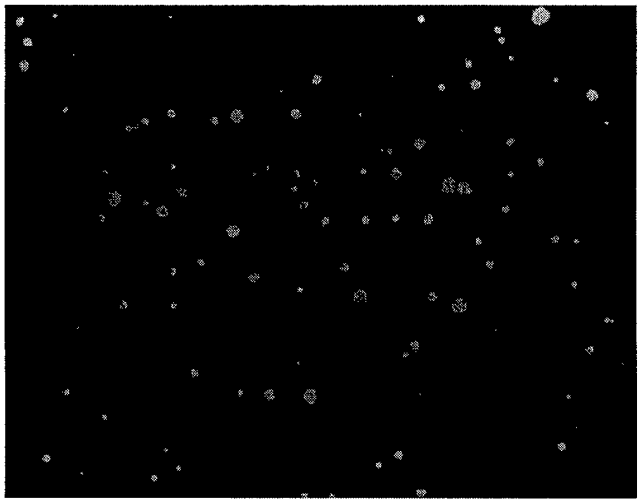

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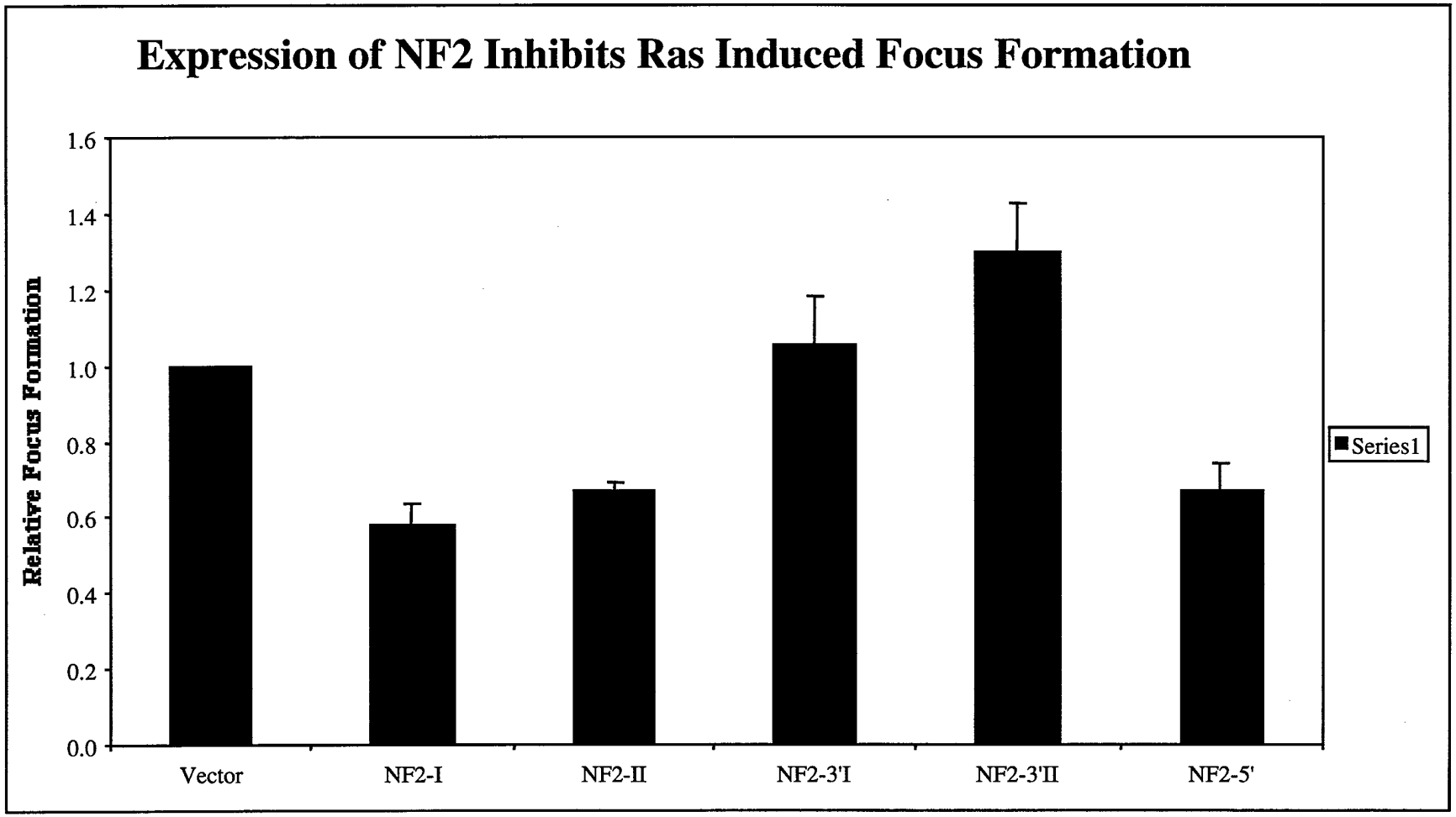
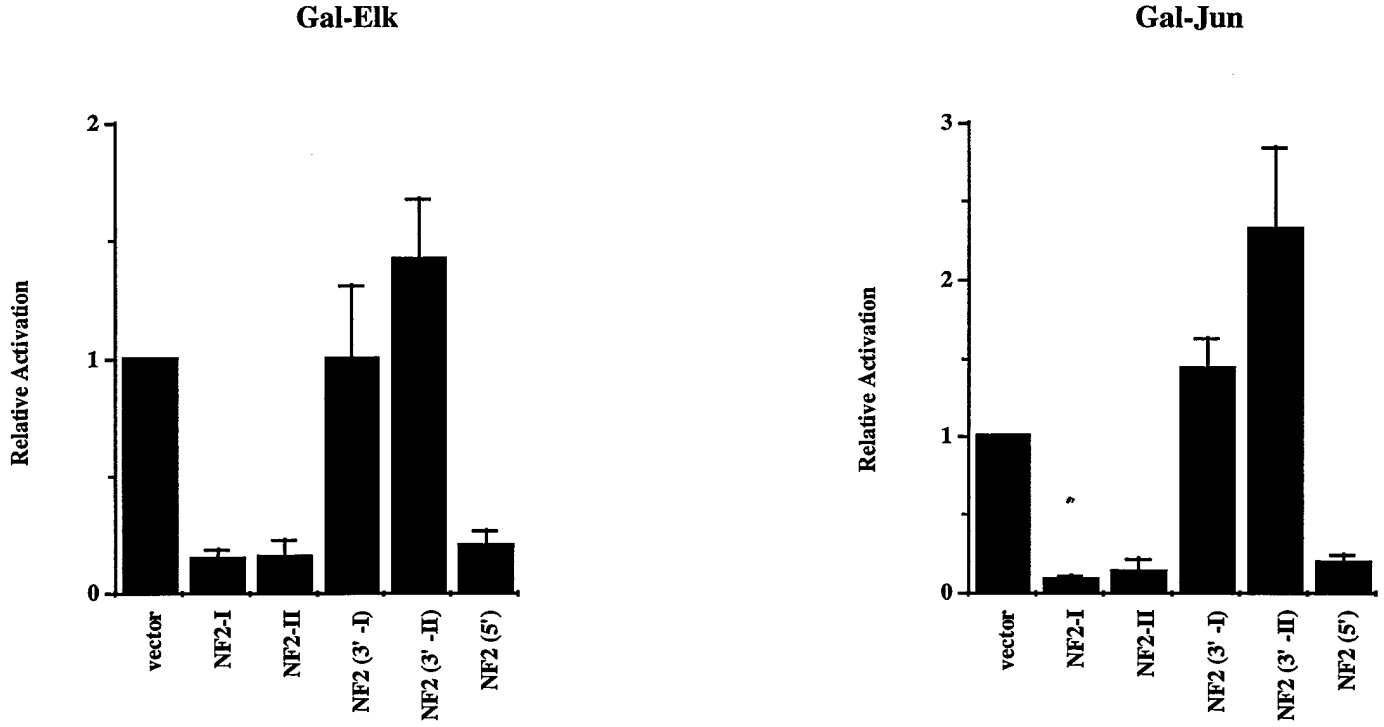


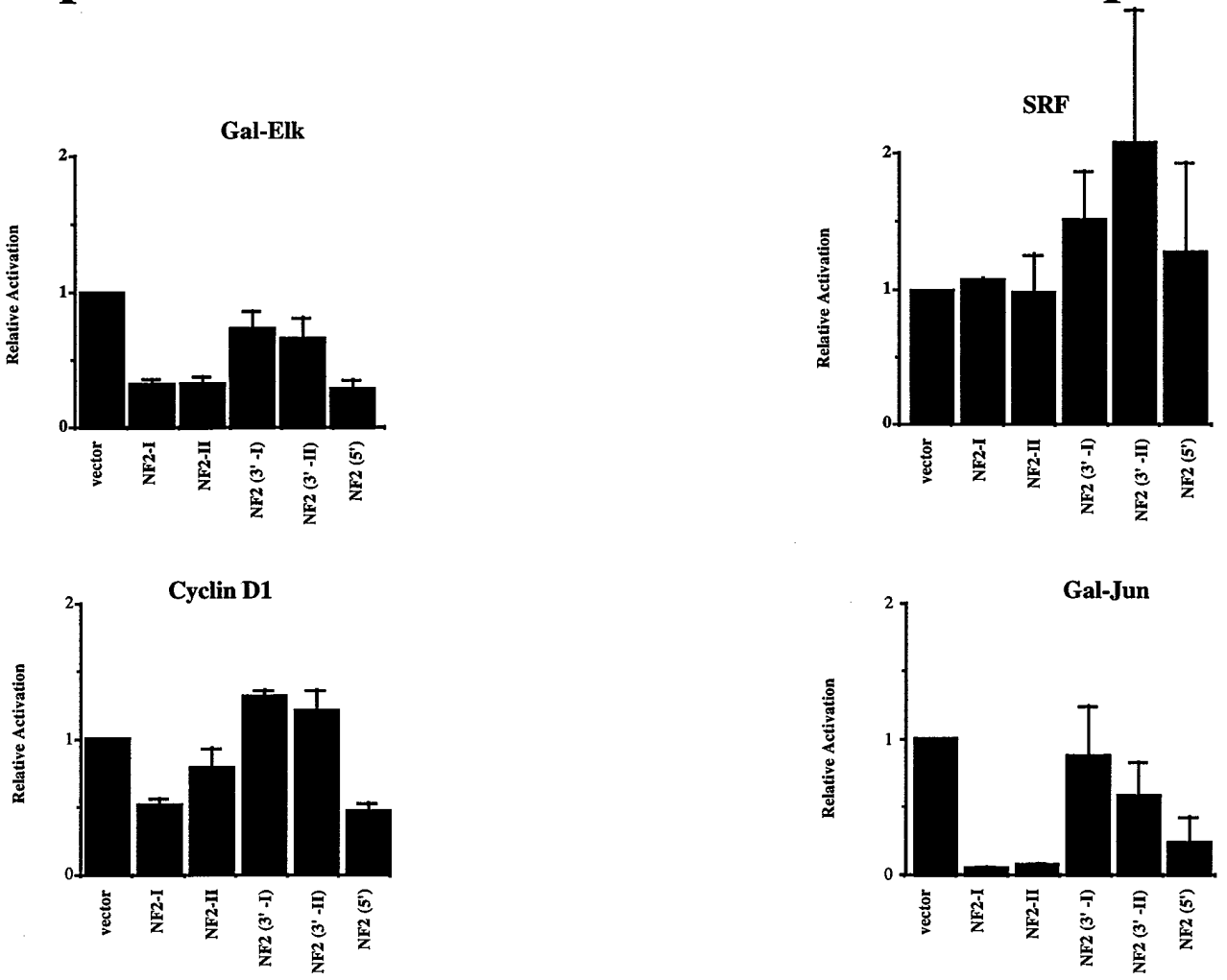
NIH/3T3 cells were transfected with 10 ng Ras along with 1 ug of each of the indicated NF2 constructs. Focus number was scored after 2 weeks. Results are the average of 3 independent experiments each done in triplicate. Standard errors are indicated by bars.

NF2 expression inhibits H-Ras61L activation of signal transduction pathways



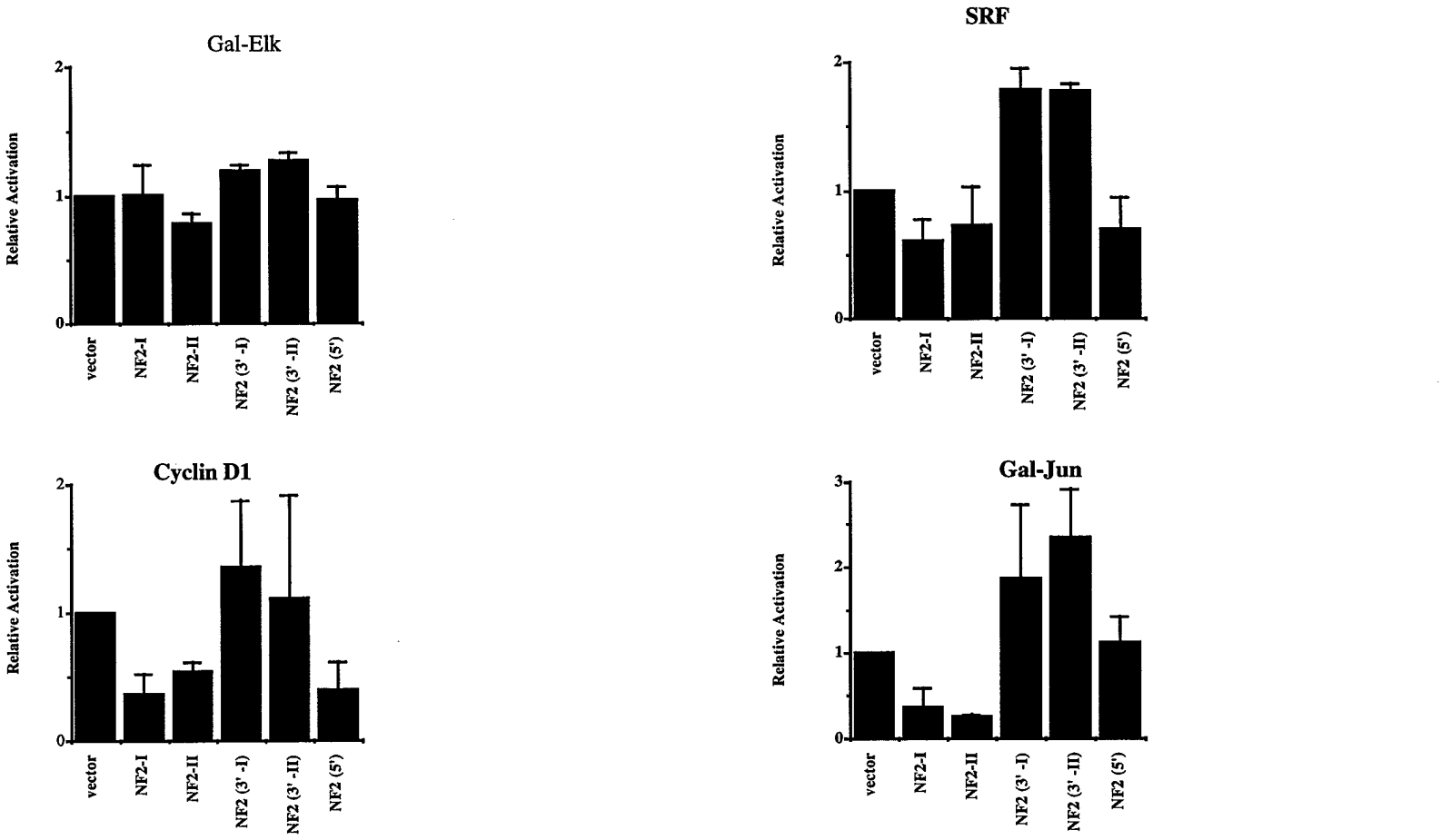
MAPK and Jnk activation was measured using the transient luciferase activity with either the Gal-Elk or Gal-Jun and 5xGal luciferase reporter systems. NIH/3T3 cells were transfected with either 10 ng/well pCGN Ras61L (Elk assay) or 0.5 ug/well pCGN Rac61L along with the various NF2 expression constructs (0.5 ug per well). Cells were serum starved ON in 0.5% CS and then luciferase activity was measured on the following day. The results shown represent the average of three independent experiments done in duplicate. Standard errors are indicated with bars.

NF2 expression inhibits serum-activated transcription pathways



NIH/3T3 cells were transfected with the indicated reported constructs along with the NF2 expression plasmids. On the 2nd day following transfection, cells were lysed and analyzed for luciferase activity in the presence of 10 % serum. Results are the average of at least 3 independent experiments each done in duplicate. Standard errors are indicated with bars.

NF2 expression inhibits basal transcription pathways in serum starved cells



NIH/3T3 cells were transfected with the indicated reported constructs along with the NF2 expression plasmids. Cells were then serum starved ON in 0.5% CS. On the 2nd day following transfection, cells were lysed and analyzed for luciferase activity. Results are the average of at least 3 independent experiments each done in duplicate. Standard errors are indicated with bars.